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Synthesis and Utility of 5-Thiocyanato Deoxyuridine and Uridine Phosphoramidites as Masked Synthons

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Abstract: *5-Thiocyanato-2'-deoxyuridine and 5-thiocyanato-uridine phosphoramidites have been synthesized and incorporated into DNA and RNA trimers via automated methodology. The SCN group has been removed with DTT treatment, and a photocrosslinking aryl azide has been added to the DNA trinucleotide through the resulting thiol group.*

The synthesis of oligoribonucleotides and oligodeoxyribonucleotides that contain modified nucleotides at specific positions provides powerful tools for the analysis of protein-nucleic acid or nucleic acid - nucleic acid interactions. Nucleotide analogs can be introduced either enzymatically, utilizing DNA and RNA polymerases, or chemically, utilizing manual or automated synthesis. Preparation of such oligonucleotides by automated synthesis, utilizing phosphoramidite nucleotides, allows for incorporation of a broad range of nucleotide analogs, without the restraints for specific substrate conformation imposed by most polymerases. Often, nucleotide analogs containing photoreactive crosslinking groups are introduced into oligonucleotides to probe protein-nucleic acid interactions via photocrosslinking (for partial review, see ref. 1). Deoxyoligonucleotides containing 4-thiothymidine or 6-thiodeoxyguanosine have been prepared and used for photochemical crosslinking of proteins directly to the nucleotide bases.[2] Similarly, oligonucleotides containing 5-(aminopropyl)-2'-deoxyuridine have been prepared and the amino group subsequently modified with fluorescent or photoactive groups.[3] We have developed a series of phosphoramidite nucleotide analogs, both ribonucleotides and deoxynucleotides, that contain masked thiol groups on base positions not involved in Watson-Crick basepairing. These analogs can be incorporated into oligonucleotides via automated synthesis and isolated with the thiol-protecting group intact. After removal of the protecting group many types of functional groups, such as photocrosslinking or fluorescent tags, can be added by utilizing thiol-modifying reagents. This adds a level of specificity to the oligonucleotide modification not present with the amino-tagged analogs previously described[3], and enables molecular interactions with the oligonucleotide that are not directly at the nucleotide base to be examined by placing functional groups at varying distances.

We report here the synthesis, incorporation into trinucleotides, and modification with an aryl azide, for the uridine versions of these analogs. Synthesis of 5-SCN-dU (**1**) was achieved using a modification of a published method[4] (Figure 1). Lead thiocyanate (1.42 g) was added to a chlorine-saturated glacial acetic acid solution (10 mL) and the reaction was stirred with a slow active addition of chlorine gas for 1.5 hours, at which time the mixture can be filtered, or used without further purification. After repeatedly de-gassing the resulting solution (SCNCl), 2'-deoxyuridine (1.0 g) was added and the reaction stirred at room temperature for 4 hours. An excess of cyclohexene (5 mL, to quench any remaining SCNCl) was added to the reaction and allowed to stir for thirty minutes. After removing the solvents and organic residues *in vacuo*, the residue was taken up in a minimum amount of acetonitrile, frozen and lyophilized overnight. Separation by HPLC gave 58 % yield of 5-thiocyanato-2'-deoxyuridine (**1**). The corresponding synthesis using 2'-deoxyuridine-5'-monophosphate proved to be unworkable, giving an intractable mixture of products.

The 5-SCN-2'-dU was further protected for automated DNA synthesis. *bis*(4-Methoxyphenyl)phenyl methyl chloride (DMTrCl, 0.6 g in 15 mL pyridine) was added dropwise to a suspension of 5-SCN-dU (0.5 g) in anhydrous pyridine, and the reaction was stirred for 18 hours. HPLC purification gave 84% product (3) after repeated lyophilization from acetonitrile. Use of acetonitrile as solvent and N,N-dimethylaminopyridine (DMAP) as base (3-5 mol eq.) resulted in slight improvement of the yield (88%), and much shorter reaction time (2.5 hrs).[5] The reaction product was purified by HPLC and lyophilized overnight. The final step was addition of 2-cyanoethyl-*N,N*-diisopropyl (CED) chlorophosphoramidite. Materials and glassware should be dry, and all steps should be accomplished under inert atmosphere. Oven dried 5-SCN-5'-DMTr-2'-dU (0.05 g) and anhydrous ethyldiisopropyl amine were dissolved in anhydrous dichloromethane (5 mL). CED (chloro) phosphoramidite (0.178 g) was added *via* syringe, and the reaction was stirred in a nitrogen atmosphere for 5 hours. After removal of solvents *in vacuo*, the residue was lyophilized over night. Exposure to atmosphere for short times is acceptable, but must be minimized for efficacy of the automated synthesis. The 5-SCN-5'-DMTr-2'-dU CED phosphoramidite (5) was then taken up in a small quantity (~1 mL) of anhydrous acetonitrile, and appropriate dilutions made for determination of concentration (λ_{max} 273 nm, ε = 10400 $M^{-1}cm^{-1}$). The phosphoramidite may be stored for some time as a dry solid under a positive pressure of inert atmosphere (nitrogen or argon) in a tightly sealed container at low temperature.

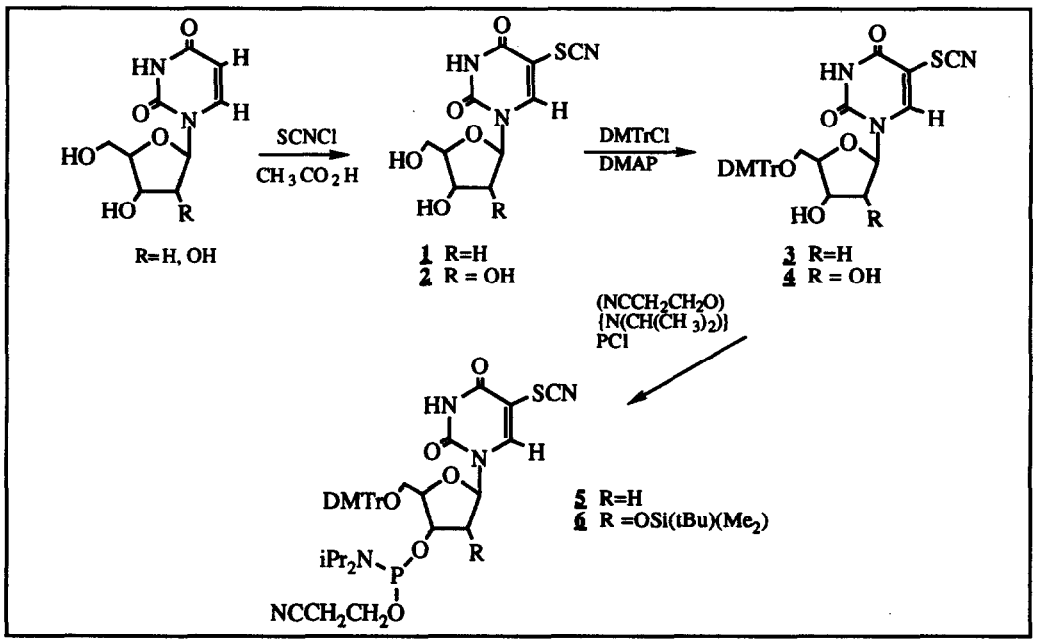

FIGURE 1: Synthesis of CED phosphoramidites of uridine and deoxyuridine.
DMTr = 4, 4'- Dimethoxy Trityl; DMAP = 4-(N,N-Dimethyl)-aminopyridine

All of these reactions have also been carried out with uridine, with similar results; the difference being the insertion of the 2'-hydroxyl protection step.[6-8] After protection of the 5' OH group with DMTr, the 5-SCN-5'-DMTr-U and silver nitrate (1.5 eq.) was dissolved in pyridine (5.5 mol equivalents) and stirred for 5-10 minutes (until the $AgNO_3$ is dissolved). *tert*-Butyldimethylsilyl chloride (TBDMSCl) was added all at once and the mixture stirred at room temperature for 1.5 hours. The reaction mixture was filtered into 5% sodium bicarbonate

solution (to prevent de-tritylation during workup), extracted with methylene chloride, and evaporated to dryness. The residue was taken up in acetonitrile and purified by HPLC.

Deprotection of 5-SCN-uridine and 5-SCN-2'-deoxyuridine with 1 mol equivalent DTT at pH 8.9 (50 mM TrisHCl) over 2 minutes[4] gave little deprotection, as shown by UV absorption at 328 nm ($\epsilon = 8.82 \times 10^{-3}$ $M^{-1}cm^{-1}$)[9] characteristic of the thiol group (Table 1). Two mol equivalents DTT and warming at 37°C for 30 minutes gave only 53% deprotection. However, nearly quantitative deprotection was observed when 3 mol equivalents DTT and warming to 55 °C for 15 minutes was employed. Similar reduction yields were achieved with the ribonucleotide analog (not shown).

TABLE 1: Effects of DTT on deoxyuridine and 5-thiocyanato-deoxyuridine

Substrate	eq DTT	Temp (°C)	Time (min)	% Reduction
Deoxyuridine	1	25	2	0
5-SCN Deoxyuridine	1	25	2	5
Deoxyuridine	2	37	30	0
5-SCN Deoxyuridine	2	37	30	53
Deoxyuridine	3	55	15	0
5-SCN Deoxyuridine	3	55	15	>95

Automated incorporation of the nucleotide analogs into trinucleotides utilizing an Applied Biosystems DNA/RNA Synthesizer (Model 392) was accomplished for both the 2'-deoxyuridine and uridine thiocyanato phosphoramidites (Figure 2). The resulting trinucleotides were cleaved from the columns, the thiocyanato

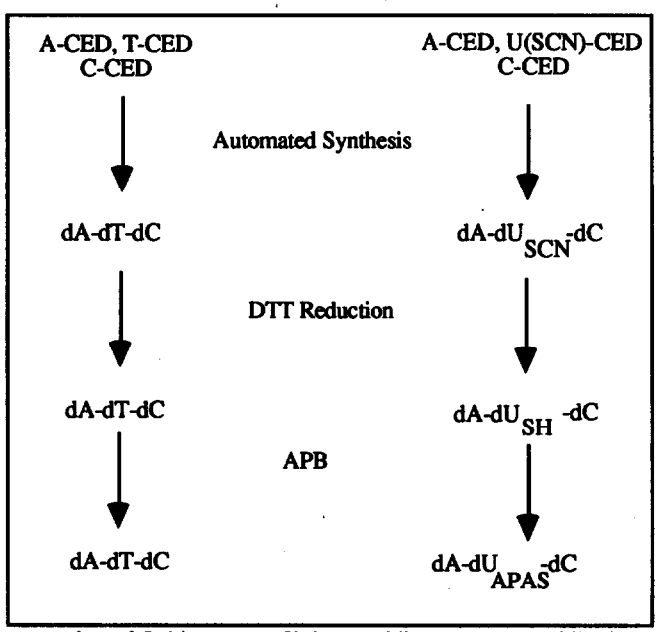

Figure 2: Incorporation of 5-thiocyanato-2'-deoxyuridine phorphoramidite into a trimer, deprotection of the oligonucleotide and modification with an aryl azide photocrosslinking group. dA = 2'-dA^{bz} CED; dT = 2'-dT CED; dU_{SCN} = 5-SCN-2'-dU CED; dU_{SH} = 5-SH-2'-dU; dU_{APAS} = 5-thio-(*S* -4-azidophenacyl)-2'-dU; dC = 2'-dC CED; DTT = dithiothreitol; APB = *p*-azidophenacylbromide.

groups were removed with DTT, and a photoreactive aryl azide group was coupled to the thiol group. Starting with a 1 micromole column, the yield of trinucleotide containing 5-SCN-deoxyuridine was 92% after cleavage from the column. Presence of the thiocyanato group after the ammonia treatment required for removal of the oligonucleotide from the column was verified by IR. Again, quantitative reduction of the thiocyanato group was achieved with 3 mol equivalents of DTT at 55° C for 15 minutes. The trimer dA-dU$_{SH}$-dC was isolated by HPLC and lyophilized for 72 hours (85% yield). DTT had no effect on the integrity of the control trimer within the detection limits of diode array UV spectral analysis. Similar yields were obtained for a trinucleotide containing 5-SCN-uridine (A-U$_{SCN}$-G). Addition of a photocrosslinking aryl azide to the deoxy-trinucleotide was accomplished by the addition of *p*-azidophenacyl bromide to a solution of the HPLC-isolated reduced trimer in 50 mM TEAB pH 8 buffer in dim or red light. Concentration of the resulting dA-dU$_{APAS}$-dG was determined by UV absorption of the azide at 300 nm (ε=20000 $M^{-1}cm^{-1}$). Nearly quantitative modification of the thiol group can be achieved with 2 to 5 equivalents of azidophenacyl bromide. Fewer than 2 equivalents results in only slightly more than 50% modification.

We have also synthesized a larger oligonucleotide (31-mer) with the deoxyuridine phosphoramidite. After reduction of the thiocyanato group, we have added a sulfur-specific fluorescent tag (5-iodoacetimidofluoresceine) to the oligonucleotide for use in protein-nucleic acid binding interaction studies. We have also radioactively labeled the oligonucleotide, attached the aryl azide crosslinker, and shown crosslinking to the *E. coli* SSB protein (single-stranded DNA binding protein, not shown). A variety of other functional groups could be attached after deprotection of the oligonucleotide. Coordination to a heavy metal atom is a possibility, for use in electron microscopy studies. The applications of this sulfur-containing analog are wide and various. We are currently carrying out synthesis of the related thiocyanato analogs of both the deoxy and ribonucleosides adenosine, guanosine, and cytidine. The synthetic pathway(s) are somewhat different. Addition of the thiocyanate ion to the 5-methyl position of thymidine would yield a reactive moiety one carbon further removed from the base than with the 5-SCN-deoxyuridine described in this paper. There are indications that substitution of a sulfur in the 6-position of guanosine may allow normal Watson-Crick basepairing.[10] Experiments are currently in the planning stages for synthesis of these analogs.

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